Estimation of p53 Antibodies in Malignant Effusions

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Objective: To detect the presence of antibodies against p53 protein in the sera and cytologically positive malignant effusions. **Design:** Cross-sectional study. **Place and Duration of study:** Department of Pathology, Postgraduate Medical Institute, Lahore from March 1997 to November 1999. **Patients and methods:** Forty cancer patients were selected with different types of malignancies and having cytologically positive effusions. Both sera and respective effusion fluid were collected and stored at –20°C. Anti-p53 ELISA was then carried out by using commercially available ELISA kit, according to the manufacturer's instructions. A positive p53 antibody level corresponded to the presence of antibodies against mutant p53 protein produced as a result of a mutation of p53 gene in the said cancer. **Results:** Positivity for anti-p53 antibodies was observed in 27 out of 40 sera (67.5%) and in 19 out of 40 effusions (47.5%) of patients with different types of cancers. The comparison revealed a significant difference with a p value of < 0.05. Out of these, 18 subjects had positive anti-p53 antibodies in both the sera and in respective effusion fluids, yielding an overall sensitivity of 66.6% and specificity of 92.3%. **Conclusion:** The present study demonstrates the usefulness of anti-p53 antibody estimation both in the serum and in effusions, as a marker of neoplasia and as an adjunct to conventional diagnostic cytopathological techniques especially in those tumours in which p53 gene mutations occur.

Key words: Effusions, p53, antibodies, ELISA.

Malignant neoplasms are characterized by their ability to metastasize. Such a spread frequently involves serosal surfaces, leading to the accumulation of fluid in pleural and peritoneal cavities[1-3]. Cytological diagnosis of malignant fluids is one of the most effective techniques for early detection of these malignancies[4].

Various ancillary methods for the detection of malignant cells in serous effusions have been proposed to increase the diagnostic accuracy of cytology[5, 6]. The study by Hall and associates (1991), demonstrates the possible usefulness of p53 immunolocalization in diagnostic cytopathology as a marker of neoplasia and an adjunct to conventional morphologic diagnosis. Extensive studies have systematically provided clinicopathological and molecular support for association of abnormalities in the p53 gene with carcinogenesis in various organs such as breast, pancreas, prostate, lung, colorectum and oesophagus[9-19].

Currently, p53 is considered to be the most frequently mutated gene in human cancer[20]. More than 350 independent mutations of this gene have been described, occurring in more than 35 different tumour types[21]. Taking into account the ten most frequent worldwide malignant tumours p53 alterations appear to be present in 40-45% of all tumours. Moreover, mutations of p53 gene are found in approximately one half of adult cancers [22 & 23].

Levels of p53 in transformed mammalian cells are 10-100 fold higher than those in untransformed cells. Such elevated levels may result from increase metabolic stability of p53 protein[24]. They accumulate to a higher level in the cells relative to the low levels associated with the wild type p53 protein and are detected by immunocytochemical analysis in cytological and histological materials. Tumour cells over expressing p53 may release it into the bloodstream; this leads, in some instances to a specific humoral response. Circulating antibodies against p53 have been detected in a variable proportion of patients with various types of cancers. Using specific heavy chain antibodies, Lubin et al in 1993, have shown that most of these p53 antibodies belong to IgG class. The data published by Angelopoulou and Diamandis (1993), suggested that although IgA and IgM antibodies against p53 also exist, their concentrations are much lower in comparison with the co-existing IgG antibodies.

p53 alterations can be assessed by three main approaches. The first is a molecular analysis of the p53 gene in which PCR and DNA sequencing lead to the specific identification of a mutation in a gene[9]. The second approach, which has been widely developed, is that of immunohistochemical analysis. The third approach consists of an assay of p53 antibodies found in sera of cancer patients[22]. This is based on initial results of Crawford et al who, in 1982, detected p53 antibodies in the sera of patients with breast carcinomas.

Lubin et al (1995) tested 200 sera from healthy blood donors for the presence of p53 antibodies. The mean $\pm$ S.D. obtained with all these sera was 1.1 $\pm$ S.D. 0.4. This data led to the conclusion that the prevalence of p53 antibodies in the normal population is very low, and that the ELISA can be effectively used on a population with various types of cancers. It is now known that in the serum of healthy subjects, the presence of p53 antibodies is extremely rare[36].

Since the first report on serum anti-p53 antibodies detection by Crawford et al (1982), such antibodies have been demonstrated in many types of cancers[2, 9-20, 26, 35, 37]. Moreover the study by Lai et al (1998) showed that anti-p53 antibodies were closely associated with malignant pleural effusion. Similarly, a complete correlation between the presence of p53 antibodies in patients' sera and

corresponding cyst and /or ascitic fluid was also documented[27].

In view of the use of p53-antibodies as a new tumour marker, the present study was carried out to detect anti-p53 antibodies by ELISA technique in the sera and corresponding malignant pleural or peritoneal effusions.

Materials and methods

A total of forty cases having malignant pleural and peritoneal effusions previously diagnosed and positive for malignant cells were included in this study. The cases were collected from medical, surgical, gynaecological and oncology units of Mayo Hospital, Services Hospital, Ghulab Devi Hospital and Lahore General Hospital, Lahore. The relevant clinical information was gathered from the hospital notes and the respective registrar of the ward.

About 40 – 50 ml of the fluid was collected in a clean dry container and the collected fluid was immediately transported to the pathology laboratory of Post Graduate Medical Institute, Lahore. The fluid was poured into clean, dry 15 ml centrifuge tubes. Centrifugation was carried out at 2000 rpm for 5 minutes. Three ml of the supernatant was transferred to storage cuvetts, labelled and kept at –20 ° C for the ELISA assay for p53 antibodies. Phlebotomy was also performed at the same time and 5 ml of venous blood of the same patient was drawn into a disposable syringe. It was poured in a sterilized test tube and allowed to clot. The serum was separated by centrifugation and stored at – 20 ° C for the ELISA assay for p53 antibodies.

Estimation of anti-p53 antibodies by enzyme linked immunosorbant assay (ELISA): Anti-p53 antibodies titers were estimated using commercially available anti-p53 ELISA II kit, (Pharmacell, Paris, France. Cat. # ELAP5302), according to the manufacturers instructions.

Principle of the test: The assay uses microtitre plates coated with recombinant wild-type p53 protein (to detect specific anti-p53 antibodies) or with control proteins (to detect non-specific interactions). A peroxidase-conjugated goat anti-human IgG binds anti-p53 antibodies. The specific p53/anti-p53/ conjugate complexes are revealed by addition of a peroxidase substrate (TMB) resulting in a colorimetric reaction.

1- The absorbance was read at 450 nm within 10 minutes after the addition of the stop solution. The **net absorbance** was determined by subtracting the assay blank absorbance from the sample or standard absorbance. For each serum and effusion sample, **specific signal** was obtained by the formula [p53 net absorbance] – [control proteins net absorbance]. p53 antibodies titre was determined using a calibration curve constructed with the pre-calibrated standards provided by the kit. The calibration curve was a linear regression curve (as mentioned in the manufacturer's instructions). The curve bisected the x-axis at 0. Levels of p53 antibodies were then determined from the calibration curve. The level of p53 antibo below 0.85U/ml were considered as negative, w the level above 1.15U/ml was taken as positive. ranges of value between 0.85-1.15U/ml were take probable presence of p53 antibodies.

Results

The cases included 16 males and 24 females. The m age of the patients with pleural effusion ranged from 6 14.54 while mean age of the patients with perito effusion ranged from 50 ± 12.86. The patients w randomly selected with maximum number from pati having Ca Lung followed by Ca Ovary. The frequenc underlying malignancy is shown in (Fig 1).

On gross examination, a large number of patients haemorrhagic effusion as compared with patients hav straw coloured effusions. The difference was statistic significant ($p = 0.01$).

Positivity for anti-p53 antibodies was observed in out of 40 sera (67.5%) and in 19 out of 40 effusi (47.5%) of patients with different types of cancers. comparison revealed a significant difference with a p va of < 0.05 (Table I). Out of these, 18 subjects had posi anti-p53 antibodies in both the sera and in respec effusion fluids (66.6%).

Fig 1: Frequency of underlying malignancy in cases malignant effusion

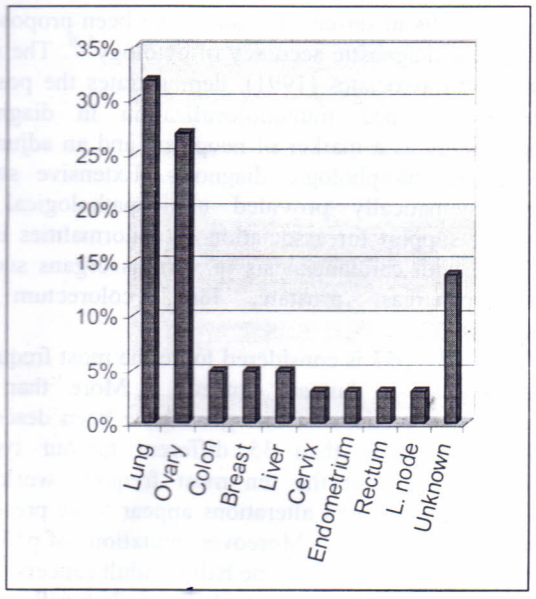

Table 1: Comparison of diagnostic value of anti-p53 ELISA cancer patients with malignant effusions

	Serum samples			
	Results	Positive	Negative	Total
Effusion Samples	Positive	18	01	19
	0	09	12	21
	Total	27	13	40

$p < 0.05$, Sensitivity = 66.6%, Specificity = 92.3%
Positive Predictive Value = 94.7%, Negative Predictive Value = 57.1%

Table 2: Comparison of diagnostic values of anti-p53 ELISA in malignant effusions

Diagnostic Procedure	Pleural effusions	Peritoneal effusions	Total effusions
Specificity	100%	87.5%	92.3%
Sensitivity	66.6%	66.6%	66.6%
P.P.V*	100%	90.9%	94.7%
N.P.V**	55.5%	58.3%	57.1%

*P.P.V Positive predictive value, **N.P.V Negative predictive value

Discussion

The detection of malignant cells in pleural, peritoneal and pericardial fluids of cancer patients marks the presence of metastatic disease and is associated with grave prognosis. Various ancillary methods have been proposed for the detection of malignant cells in serous effusions[5]. The detection of malignant cells in effusions is facilitated by the use of immunochemistry using a wide panel of antibodies[2, 29]. Serological analysis of anti-p53 antibodies has been widely employed as an alternate (or complementary) procedure with immunohistochemical staining to assess the p53 status in cancer patients. Mutation of p53 gene is a genetic alteration found in human cancers[31], and anti-p53 antibodies are autoantibodies induced by mutation of p53 gene. Thus they are considered to be the indirect markers for p53 gene mutations and abnormally increased p53 gene levels[32].

In the present study, serum and effusion p53 levels were estimated in patients with different types of malignancies. A total of 27(67.5%) out of 40 previously diagnosed cancer patients showed positive serum anti-p53 antibodies. Sakai and Okamoto[30], in their study on the serum of patients with malignant neoplasms reported that anti-p53 antibody concentration was high in patients with lung, oesophageal, gastric, hepatocellular, colonic, rectal and ovarian cancer.

Takeda et al[17], reported that serum anti-p53 antibodies were detected in 63% (17/27) patients with colorectal adenocarcinoma. A similar result was seen by Ralhan et al[18]. They observed a high prevalence, 36 out of 60(60%), of circulating anti-p53 antibodies in oesophageal squamous cell carcinoma.

Many studies have been conducted to estimate the anti-p53 antibody status in serous effusions and led to variable results. In our study, a total of 19, out of 40 (47%) malignant effusions were positive for anti-p53 antibodies and in 66.6% (18/27) cases had anti-p53 antibodies present both in the serum and in the effusion fluid respectively.

Zoppi et al[31] reported a positive rate of 32.4% in malignant effusions. They assessed the immunohistochemical determination of p53 antibody in 34 embedded blocks of neoplastic fluids and 30 non-neoplastic effusions. Similarly, the study by Montenarh et al[27], showed that nearly 8.7% of patients with ovarian cancer had antibodies against p53 and these antibodies can be detected in the sera as well as in cyst and in ascitic fluids by immunohistochemistry, Immuno-blot and Elisa procedures. A similar study was conducted by Abendenstein et al[32], who reported 21% positivity of p53 antibodies in ascitic fluid of patients with epithelial ovarian carcinoma.

The difference in the positivity rates in p53 antibody estimation is no exception as it is known that the frequency of p53 antibodies vary from study to study even for a given type of cancer[27]. Moreover, increased concentration of p53 antibodies were seen in patients with lung and ovarian cancers[32], and our study mainly comprised of patients with lung and ovarian cancers.

Conclusion

The present study thus demonstrates the usefulness of anti-p53 antibody estimation both in the serum and in effusions, as a marker of neoplasia and as an adjunct to conventional diagnostic cytopathological techniques especially in those tumours in which p53 gene mutations occur. Anti-p53 ELISA is a highly specific, moderately sensitive procedure for the detection of p53 antibodies, both in the sera and in the effusion of cancer patients. It is further recommended that larger study may be carried out on benign and malignant effusions to find out the base line for p53 antibodies.

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